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The C-Terminal Region of Rift Valley Fever Virus NSm Protein Targets the Protein to the Mitochondrial Outer Membrane and Exerts Antiapoptotic Function

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The NSm nonstructural protein of Rift Valley fever virus (family *Bunyaviridae*, genus *Phlebovirus*) has an antiapoptotic function and affects viral pathogenesis. We found that NSm integrates into the mitochondrial outer membrane and that the protein's N terminus is exposed to the cytoplasm. The C-terminal region of NSm, which contains a basic amino acid cluster and a putative transmembrane domain, targeted the protein to the mitochondrial outer membrane and exerted antiapoptotic function.

Rift Valley fever virus (RVFV) (family *Bunyaviridae*, genus *Phlebovirus*) causes a mosquito-borne disease characterized by febrile illness and high abortion rates in ruminants and an acute febrile illness that may be followed by fatal hemorrhagic fever, encephalitis, or ocular disease in humans (1, 2). RVFV is endemic to sub-Saharan African countries, but outbreaks also have occurred in Egypt, Saudi Arabia, and Yemen (3). Because many different competent mosquito vector species are present in other geographic areas (4), there is serious concern regarding the spread of RVFV to other areas of the world.

RVFV has a single-stranded, tripartite RNA genome composed of large (L), medium (M), and small (S) RNA segments. The antiviral sense L RNA encodes the viral RNA-dependent RNA polymerase, while the antiviral sense M RNA encodes two major envelope glycoproteins, Gn and Gc, and two accessory proteins, the NSm protein, a 14-kDa nonstructural protein, and the 78-kDa protein, a minor viral structural protein (5, 6). The open reading frame of the M mRNA contains five in-frame translation initiation codons within the pre-Gn region, located upstream of the Gn and Gc genes; the Gn and Gc proteins are translated from 4th and 5th AUGs (Fig. 1A) (7). NSm is translated from the 2nd AUG, and its C-terminal region is thought to be generated by signal peptidase-mediated cleavage at the N terminus of the Gn coding region (7). The 78-kDa protein is translated from the 1st AUG, and its coding sequence includes the entire NSm and Gn coding sequences (7). The S RNA uses an ambisense strategy to express the N protein and an accessory protein, NSs (5).

RVFV NSm is not essential for viral replication in cell culture (8), although it regulates the p38 mitogen-activated protein kinase response in mammalian cells (9) and is important for virus infectivity in *Aedes aegypti* mosquitoes (10). We reported that an RVFV mutant lacking expression of both the 78-kDa and NSm proteins induced more extensive apoptosis, including efficient cleavage of caspases 3/7, 8, and 9, than did the wild-type RVFV (11). Expressed NSm was also shown to inhibit staurosporine (STP)-induced cleavage of caspases 8 and 9 (11), demonstrating that the NSm protein has an antiapoptotic function. The region or regions of NSm that are required for regulation of the p38 mitogen-activated protein kinase response, infectivity in mosquitoes, and antiapoptosis function have not been identified yet. An RVFV mutant lacking the NSm gene showed decreased virulence in a rat model compared to wild-type RVFV (12), implying that the antiapoptotic function of NSm plays a role in viral pathogenicity.

We examined the subcellular localization of NSm to investigate the mechanism of NSm-mediated apoptosis suppression. 293 cells inoculated with arMP-12 (Fig. 1B), an attenuated RVFV strain rescued from cDNAs (13), were immunostained with a rabbit anti-NSm peptide antibody that was raised against a 13-amino-acid synthetic peptide (HGKDPEDKISLIKG) and recognizes both the NSm and 78-kDa proteins and an antibody recognizing either an integral mitochondrial outer membrane (MOM) protein, Tom20, or an endoplasmic reticulum marker, calreticulin, followed by incubation with Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Viral proteins recognized by the anti-NSm peptide antibody colocalized with Tom20, but not with calreticulin (Fig. 1C). Subcellular fractionation analysis also showed the presence of NSm in the mitochondrial fraction (Fig. 1D), demonstrating the mitochondrial localization of NSm in infected cells.

To unambiguously identify the subcellular localization of the NSm protein using microscopic analysis, we generated a new virus, delM-S-V5-NSm (Fig. 1B), with a deletion in the pre-Gn region of the M RNA from nucleotides (nt) 21 to 384 and an N-terminal V5 epitope-tagged NSm gene in place of the NSs gene in the S RNA; this virus does not express the NSm or 78-kDa proteins from the M RNA, but rather it expresses the NSm protein carrying a N-terminal V5 tag (V5-NSm) from the S RNA. We also generated a mutant virus encoding an N-terminal V5-tagged NSm from the 2nd AUG and lacking 78-kDa protein expression by removing the 1st AUG in the M gene open reading frame. However, this mutant virus was not suitable for the present study due to the poor accumulation of the V5-tagged NSm protein in infected cells (data not shown). In delM-S-V5-NSm-infected cells, V5-NSm colocal-

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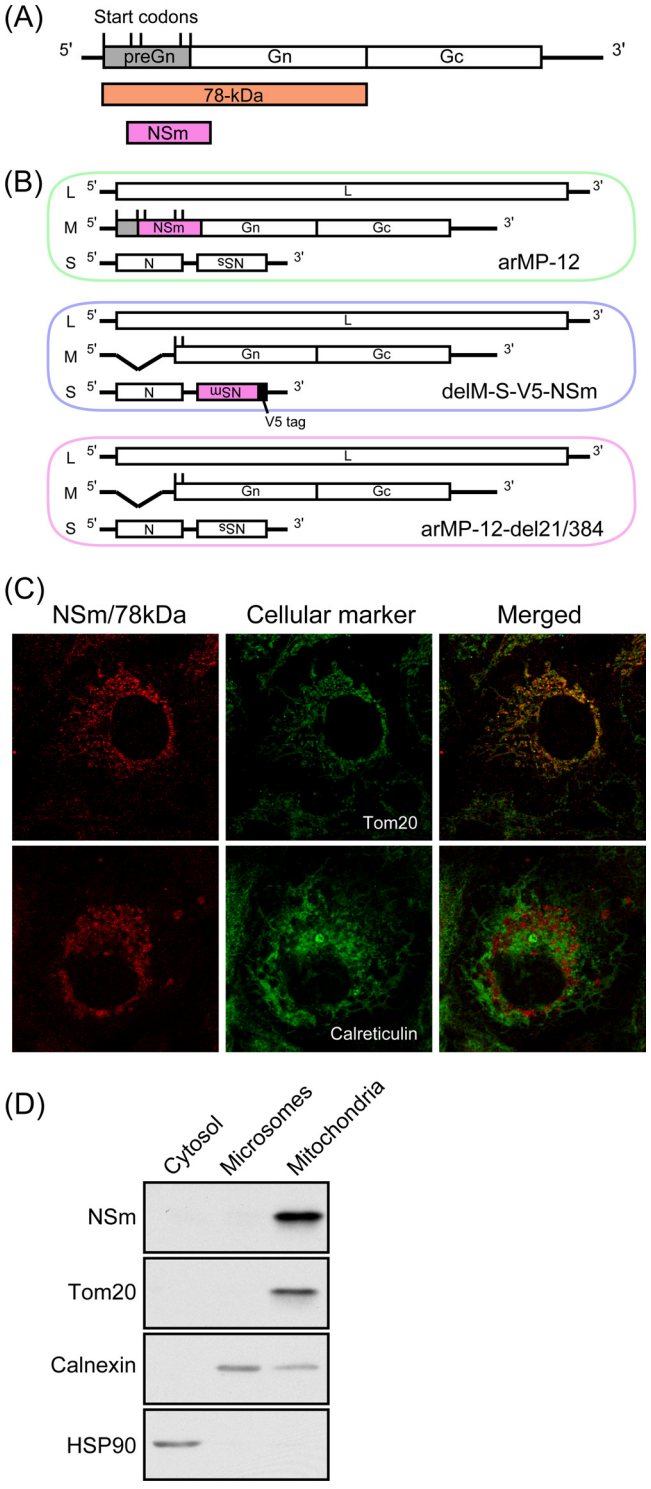

FIG 1 (A) Schematic diagram of the anti-genomic-sense M segment of RVFV and coding regions of NSm, 78-kDa, Gn, and Gc proteins. The five in-frame translation initiation codons present in the pre-Gn region are illustrated by five short vertical lines. (B) Schematic diagram of the anti-genomic-sense genome of arMP-12, delM-S-V5-NSm, and arMP-12-del21/384. L, M, and S represent the anti-genomic-sense L, M, and S RNAs, respectively. (C) Confocal microscopic analysis of the subcellular localization of NSm in arMP-12-infected 293 cells. 293 cells grown on 8-well Lab-Tek II chamber slides (Nalgene Nunc International) were inoculated with arMP-12 at a multiplicity of infection (MOI) of 1. At 8 h postinoculation (p.i.), the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin, and immunostained with a rabbit anti-NSm peptide antibody and a mouse anti-Tom20 monoclonal antibody (Abcam) (top panels) or a rabbit anti-NSm peptide antibody and a chicken anti-calreticulin polyclonal antibody (Abcam) (bottom panels), followed by incubation with Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Images were captured by Zeiss LSM 510 confocal laser-scanning microscopy with a 63× oil immersion lens and processed with the LSM image browser and ImageJ software (23). (D) Cell extracts of 293 cells infected with arMP-12 at an MOI of 1 were collected at 16 h p.i. and fractionated by Percoll gradient centrifugation (24). The distribution of NSm was examined by Western blotting using an anti-NSm antibody (8), and the purity of each fraction was examined by Western blotting using a rabbit anti-HSP90 polyclonal antibody (Cell Signaling Technology), a goat anti-calnexin polyclonal antibody (Santa Cruz Biotechnology), and an anti-Tom20 monoclonal antibody.

ized with Tom20, and both proteins had similar fluorescence histogram patterns (Fig. 2A), demonstrating the localization of V5-NSm in the MOM. In contrast, V5-NSm did not colocalize with calreticulin, and the fluorescence histogram patterns of V5-NSm and SDHA, a marker for the mitochondrial inner membrane, did not match. Expressed V5-NSm also colocalized with MOM, but not with SDHA or calreticulin (Fig. 2B), thereby demonstrating that other viral proteins are not required for NSm to target the MOM.

The nature of the NSm-MOM interaction was examined next. To determine the membrane topology of NSm, mitochondrial fractions isolated from delM-S-V5-NSm-infected cells were incubated with proteinase K and subjected to Western blot analysis. As expected, an anti-Tom20 antibody, which binds to the cytoplasmic domain of Tom20, detected Tom20 in the absence of proteinase K treatment, but not after proteinase K treatment. An anti-V5 antibody detected the V5-NSm signal in the proteinase K-untreated sample, but not in the proteinase K-treated sample (Fig. 2C). Cytochrome *c*, which is located in the mitochondrial intermembrane space (14), was resistant to proteinase K treatment, confirming that the proteinase K treatment did not disrupt the mitochondrial membranes. Following permeabilization of the membranes by detergent treatment, all of the above proteins were susceptible to protease digestion (Fig. 2C). These data indicate that the N-terminal V5 tag is localized to the cytoplasm. To investigate whether NSm is an integral membrane protein, the mitochondrial fractions from delM-S-V5-NSm-infected cells were subjected to an alkali extraction in which only integral membrane proteins are pelleted down by centrifugation following alkali treatment (15). Consistent with our expectation, Tom20, but not cytochrome *c*, was detected in the pellet (Fig. 2D). We also detected V5-NSm in the pellet, thus demonstrating that NSm is an integral protein (Fig. 2D). From this, we concluded that NSm is an integral MOM protein with its N terminus exposed to the cytoplasm.

NSm is ~115 amino acids long and has a hydrophobic amino acid cluster in its C-terminal region (Fig. 3A). The TMpred program (16) predicted two possible transmembrane helices at amino acids 95 to 112 and 99 to 115, implying that the C-terminal hydrophobic region contains a transmembrane domain, which is inserted into the MOM. To understand the role of the C-terminal region of NSm in MOM targeting, we examined the subcellular localizations of expressed fusion proteins comprised of an N-terminal Venus protein and a variable-length NSm-derived C terminus; Venus-NSm71-115, Venus-NSm86-115, and Venus-NSm93-115 had a C terminus corre-

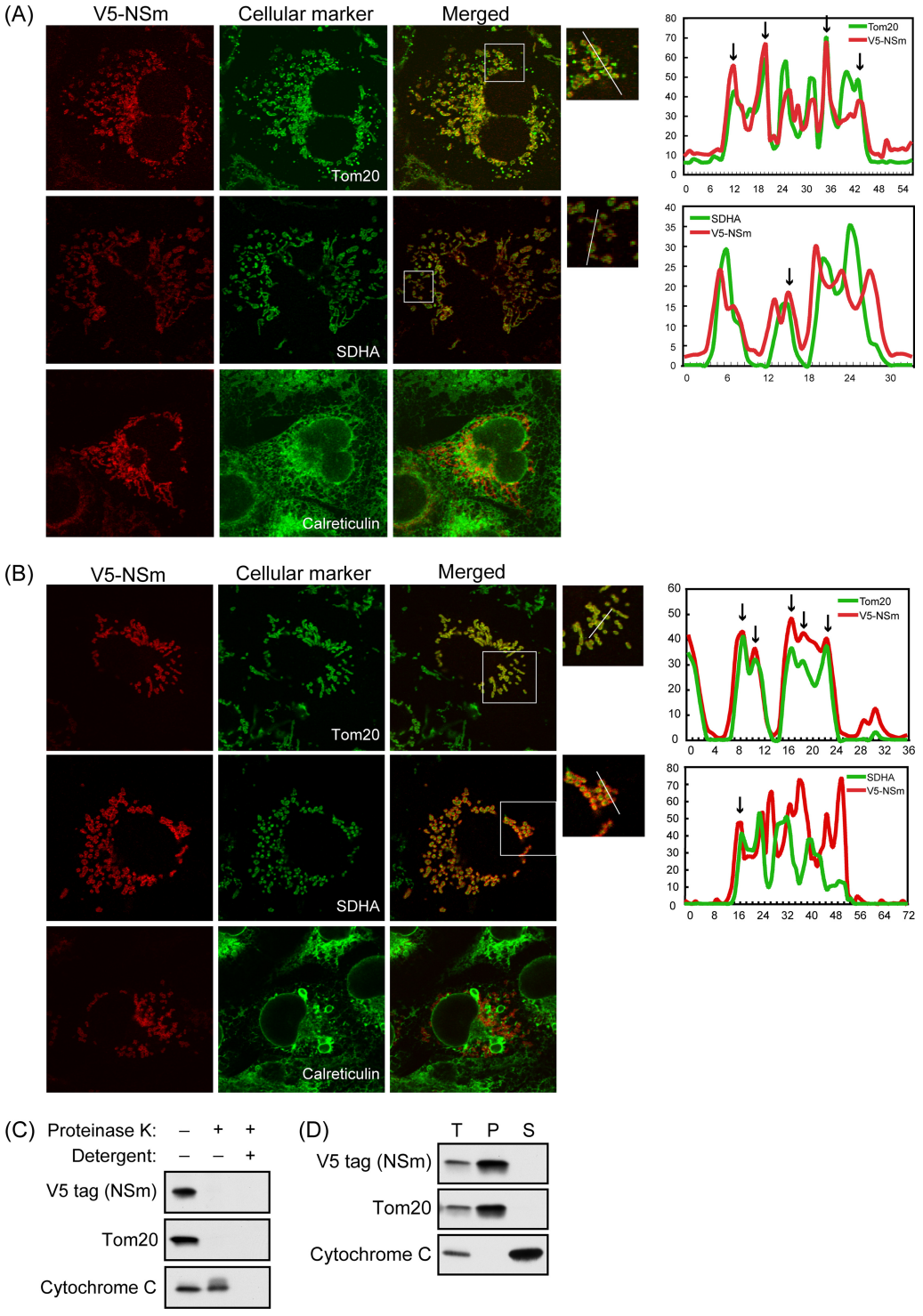

FIG 2 NSm is an integral membrane protein anchored in the MOM. (A) 293 cells infected with delM-S-V5-NSm at an MOI of 0.5 were fixed at 8 h p.i. and costained with a rabbit anti-V5 tag polyclonal antibody (Abcam) and an antibody specific for the mitochondrial inner membrane protein SDHA (clone 2E3; Abcam), the mitochondrial outer membrane protein Tom20, or the endoplasmic reticulum protein calreticulin. Histograms display the fluorescence signal intensities along the white line in the small square panels of V5-NSm (red) and Tom20 (green) in the upper panel and V5-NSm (red) and SDHA (green) in the lower panel. The small square panels to the right of the merged images correspond to the indicated regions in the merged images. The *x* and *y* axes in the histograms represent the distance from one end of the line in arbitrary units and signal intensity in an arbitrary scale, respectively. Arrows in the histograms indicate the fluorescent intensity peaks of V5-NSm and those of the cellular markers, which are precisely matched. (B) Experiments were performed as described in panel A, except 293 cells were transfected with an expression plasmid encoding V5-NSm and the cells were fixed at 8 h posttransfection. (C) Crude mitochondrial fractions were obtained from delM-S-V5-NSm-infected 293 cells (MOI of 0.5) at 16 h p.i. Cells were left untreated (left lanes), treated with 150 μg/ml of proteinase K (middle lanes), or treated first with detergents (1% Triton X-100 and 1% SDS) and then incubated with proteinase K (right lanes), according to the previously described method (25, 26). The samples were analyzed by Western blotting with an anti-V5 antibody or antibodies against the mitochondrial marker proteins. (D) Crude mitochondrial fractions were obtained from delM-S-V5-NSm-infected 293 cells (MOI of 0.5) at 16 h p.i., treated with 0.1 M sodium carbonate (pH 12) for 20 min at 4°C, and subjected to ultracentrifugation at 22,300 rpm at 4°C using a Beckman SW55 Ti rotor (27). The collected insoluble pellet (P) and soluble supernatant (S) fractions were analyzed by Western blotting with an anti-V5 antibody or antibodies against the mitochondrial marker proteins. T represents the total crude mitochondrial fractions.

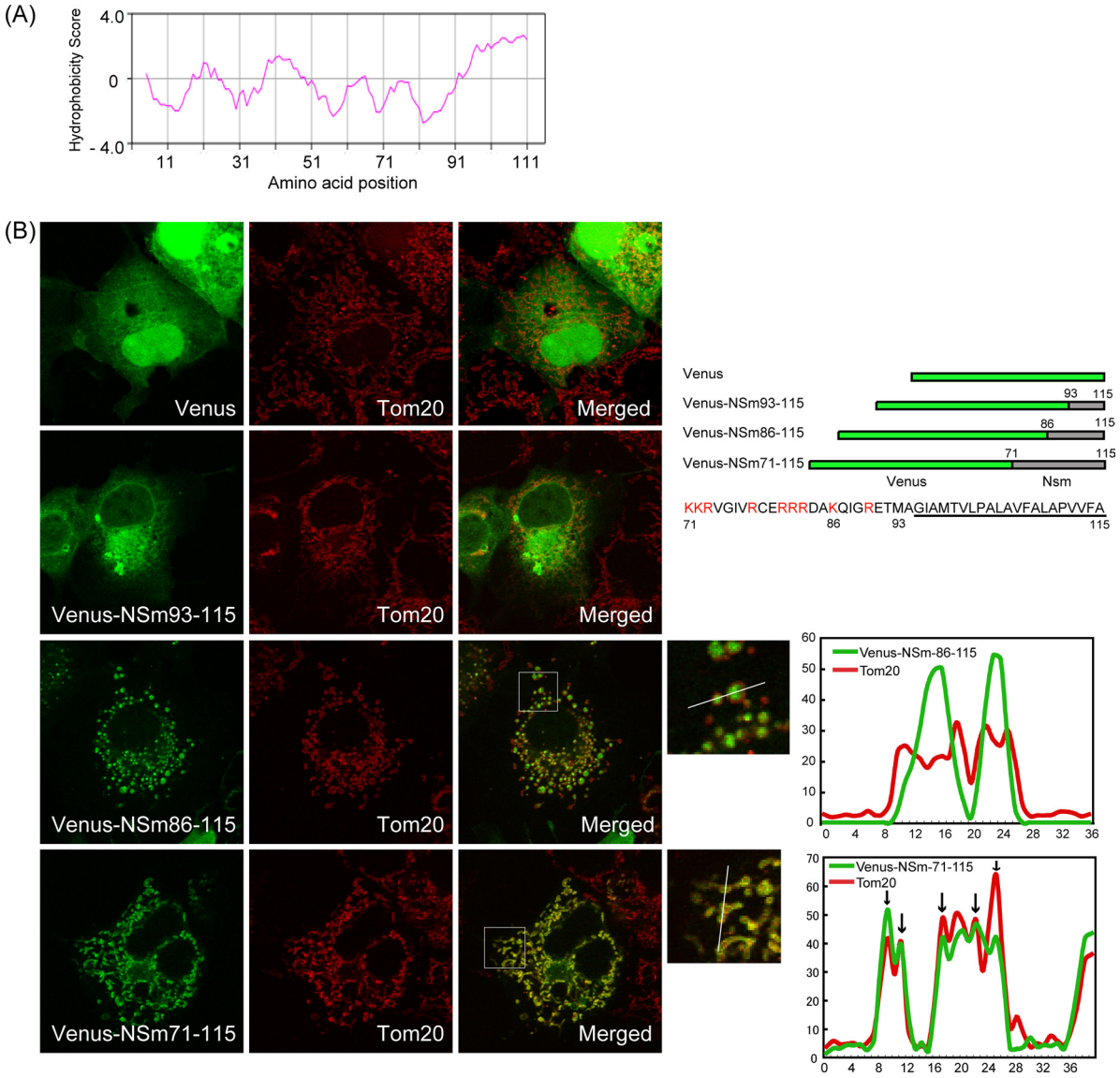

FIG 3 Identification of an NSm region that targets the protein to the MOM. (A) A Kyte-Doolittle hydropathy plot analysis (28) of NSm, using window size 9. (B) 293 cells were transfected with plasmid expressing the Venus protein or a fusion protein comprised of Venus and a variable-length portion of the NSm C-terminal region (upper right diagram,). Numbers represent the amino acid positions within the full-length NSm protein. The amino acid sequence of the C-terminal region of NSm is shown below the schematic diagram. Red letters and the underline represent basic amino acids and the putative transmembrane domain, respectively. The cells were fixed at 8 h posttransfection and stained with an anti-Tom20 antibody. The large panels on the left of the figure show the signals of Venus, Tom20, and merged images, from left to right. On the lower right, histograms display the measured fluorescence signal intensities along the white line in the small square panels for Venus-88-115 (green) and Tom20 (red) in the upper panel and Venus-71-115 (green) and Tom20 (red) in the lower panel. The *x* and *y* axes of the histograms represent the distance from one end of the line in arbitrary units and signal intensity in an arbitrary scale, respectively. Arrows in the histograms indicate the fluorescent intensity peaks of Venus and those of Tom20 that are precisely matched. The small square panels to the right of the merged images correspond to the indicated regions in the merged images.

sponding to amino acids 71 to 115, 86 to 115, and 93 to 115 of NSm, respectively (Fig. 3B). The expressed Venus protein and expressed Venus-NSm93-115, containing the putative transmembrane domain, did not colocalize with Tom20 (Fig. 3B). The Venus-NSm86-115 signal corresponded to a mitochondrial localization pattern, but it did not colocalize with Tom20. In contrast, Venus-NSm71-115 colocalized with Tom20, demonstrating that the C-terminal amino acids 71 to 115 targeted NSm to the MOM.

We next identified the NSm region that exerts antiapoptotic function. To this end, we constructed a control plasmid encoding a Venus protein mutant (V5-VeFD) with an N-terminal V5 tag and a small internal deletion that inactivated the Venus chromophore activity, thereby facilitating its use in a caspase-3 colorimetric assay. We also constructed four plasmids, each of which expressed a fusion protein comprised of an N-terminal V5-VeFD and one of the following regions from NSm: full-length NSm (V5-VeFD-NSm), amino acids 1 to 70 (V5-VeFD-NSmMOMdel), amino acids 71 to 115 (V5-VeFD-MOM), or amino acids 1 to 100 (V5-VeFD-NSm-TMdel) (Fig. 4A). The levels of accumulation of

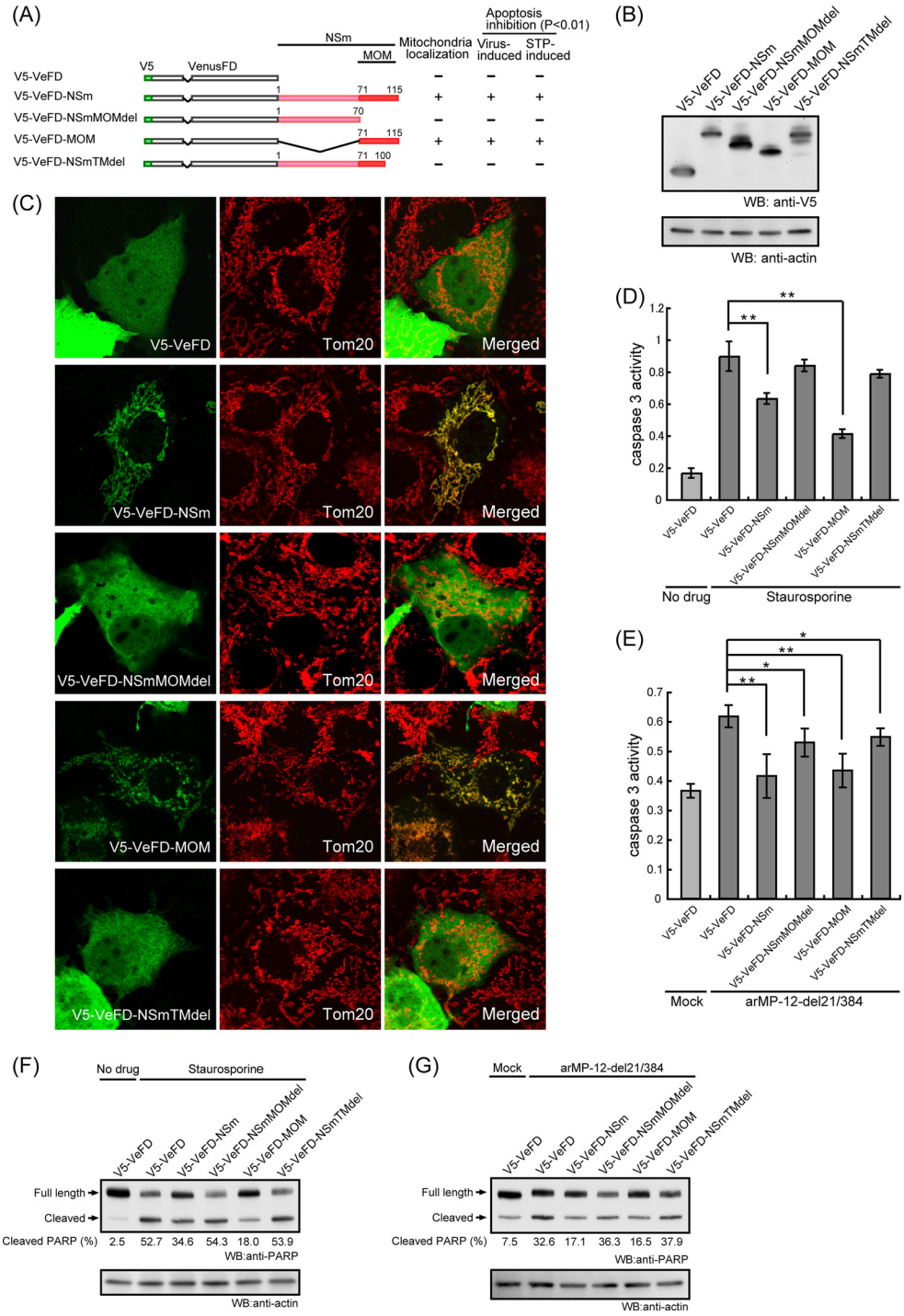

FIG 4 The MOM targeting signal of NSm exerts its antiapoptotic function. (A) Schematic diagrams of the Venus protein mutant (V5-VeFD) carrying an N-terminal V5 tag and a deletion at amino acids 65 to 69 and fusion proteins with an N-terminal V5-VeFD sequence and either a full-length NSm sequence (V5-VeFD-NSm) or another NSm-derived sequence. Numbers represent the amino acid positions in the full-length NSm protein. MOM represents the MOM targeting signal. (B) 293 cells were transfected with plasmids expressing each of proteins depicted in panel A. Cell extracts were prepared at 24 h posttransfection and subjected to Western blot (WB) analysis using an anti-V5 antibody and an antiactin antibody (Santa Cruz Biotechnology). (C) 293 cells were transfected with plasmids encoding each of the proteins depicted in panel A. At 8 h posttransfection, cells were fixed, immunostained by an anti-V5 antibody and an anti-Tom20 antibody, and subjected to confocal microscopic analysis. (D) 293 cells were transfected with plasmids encoding each of the proteins from panel A and at 16 h posttransfection were treated with 3 μM staurosporine for 3 h or left untreated (No drug). After determination of the protein concentration of the cell lysate by the Bradford assay, 100 μg of lysate was incubated with the colorimetric peptide substrate DEVD-pNA for 2 h. Color changes were measured at 405 nm, and the background reading was subtracted from the sample reading to measure caspase-3 activity. (E) 293 cells were transfected with plasmids expressing each of the proteins in panel A. At 8 h posttransfection, the cells were infected with arMP-12-del21/384 at an MOI of 3. Cell lysate was collected at 16 h p.i. and subjected to the caspase 3 assay. The data shown in panels D and E are representative of at least three independent experiments, and statistical significance was determined using unpaired Student's *t* tests (*, $P < 0.05$; **, $P < 0.01$). (F) Experiments were performed as described in panel D. The cell lysates were subjected to Western blot analysis by using an anti-PARP antibody (Cell Signaling Technology) and antiactin antibodies to determine the levels of cleavage of PARP. The specific band signal in each immunoblot was quantified by densitometric scanning, and the percentage of cleavage of PARP was calculated by dividing cleaved PARP by total PARP. (G) Experiments were performed as described in panel E. The level of cleavage of PARP was determined as described in panel F. Data shown in panels F and G are representative of at least three independent experiments.

all of the expressed proteins were comparable (Fig. 4B). Confocal microscopic analyses using an anti-V5 antibody and an anti-Tom20 antibody showed that only V5-VeFD-NSm and V5-VeFD-MOM, both of which had the MOM targeting signal, localized to the MOM (Fig. 4C). V5-VeFD-NSmMOMdel and V5-VeFD-NSmTMdel had a diffuse cytoplasmic distribution and did not colocalize with Tom20, demonstrating the requirement of the putative transmembrane region for the mitochondrial targeting of NSm. To induce apoptosis, 293 cells expressing each of these proteins were treated with STP (Sigma-Aldrich) or infected with arMP-12-del21/384, an arMP-12-derived mutant virus with a deletion in the pre-Gn region (11) (Fig. 1B), and cell extracts were subjected to a caspase-3 colorimetric assay (BioVision, Mountain View, CA). Consistent with our previous study (11), caspase-3 activity due to both STP- and arMP-12-del21/384 virus-induced apoptosis in V5-VeFD-NSm-expressing cells was statistically lower ($P < 0.01$) than in V5-VeFD-expressing cells, demonstrating that V5-VeFD-NSm inhibited both STP- and virus-induced apoptosis (Fig. 4D and E). Expressed V5-VeFD-MOM also suppressed apoptosis induced both by STP and arMP-12-del21/384 at statistically significant levels ($P < 0.01$), demonstrating that amino acids 71 to 115 of NSm are sufficient for apoptosis suppression. Expressed V5-VeFD-NSmMOMdel and V5-VeFD-NSmTMdel failed to suppress STP-induced apoptosis, although both suppressed arMP-12-del21/384-induced caspase-3 activation at modest, although statistically significant, levels ($P < 0.05$), implying that the cytoplasmic region of NSm may also moderate virus-induced apoptosis. Consistent with the caspase-3 colorimetric assay, expression of V5-VeFD-NSm or V5-VeFD-NSmMOM, but not V5-VeFD-NSmMOMdel and V5-VeFD-NSmTMdel, inhibited both STP- and arMP-12-del21/384-induced cleavage of poly(ADP-ribose) polymerase (PARP), which is a downstream substrate of caspase-3 (Fig. 4F and G).

The present study revealed the importance of the C-terminal region amino acids 71 to 115 of RVFV NSm for the targeting of the protein to the MOM and the exertion of antiapoptotic function. The targeting of tail- or tip-anchored proteins to the MOM often requires the presence of a short hydrophilic sequence, rich in basic residues, near the transmembrane domain (17–19). Venus-NSm71-115, which had 9 basic amino acids upstream of the putative transmembrane domain, but not Venus-NSm86-115, which had 2 basic amino acids upstream of the putative transmembrane domain, localized to the MOM (Fig. 3), suggesting the requirement for more than two basic amino acids upstream of the putative transmembrane domain for the targeting of NSm to the MOM. Viral proteins that target the mitochondria often have a Bcl-2 homology (BH) domain(s) and exert their antiapoptotic activities by interacting with a host proapoptotic protein through their BH domain(s) (20–22). We were unable to identify a BH domain within the entire NSm amino acid sequence. The mechanism by which the C-terminal region of NSm exerts its antiapoptotic function in the absence of a BH domain remains to be investigated.

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REFERENCES

1. **Balkhy HH, Memish ZA.** 2008. Rift Valley fever: an uninvited zoonosis in the Arabian Peninsula. Int. J. Antimicrob. Agents **21**:153–157.
2. **Peters CJ, Meegan JM.** 1989. Rift Valley fever. CRC Press, Boca Raton, FL.
3. **Bird BH, Ksiazek TG, Nichol ST, Maclachlan NJ.** 2009. Rift Valley fever virus. J. Am. Vet. Med. Assoc. **234**:883–893.
4. **Gargan TP, II, Clark GG, Dohm DJ, Turell MJ, Bailey CL.** 1988. Vector potential of selected North American mosquito species for Rift Valley fever virus. Am. J. Trop. Med. Hyg. **38**:440–446.
5. **Schmaljohn CS, Nichol ST.** 2007. Bunyaviruses, p 1741–1789. *In* Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (ed), Fields virology, 5th ed, vol 2. Lippincott, Williams & Wilkins, Philadelphia, PA.
6. **Struthers JK, Swanepoel R, Shepherd SP.** 1984. Protein synthesis in Rift Valley fever virus-infected cells. Virology **134**:118–124.
7. **Kakach LT, Suzich JA, Collett MS.** 1989. Rift Valley fever virus M segment: phlebovirus expression strategy and protein glycosylation. Virology **170**:505–510.
8. **Won S, Ikegami T, Peters CJ, Makino S.** 2006. NSm and 78-kilodalton proteins of Rift Valley fever virus are nonessential for viral replication in cell culture. J. Virol. **80**:8274–8278.
9. **Narayanan A, Popova T, Turell M, Kidd J, Chertow J, Popov SG, Bailey C, Kashanchi F, Kehn-Hall K.** 2011. Alteration in superoxide dismutase 1 causes oxidative stress and p38 MAPK activation following RVFV infection. PLoS One **6**e20354. doi:10.1371/journal.pone.0020354.
10. **Crabtree MB, Kent Crockett RJ, Bird BH, Nichol ST, Erickson BR, Biggerstaff BJ, Horiuchi K, Miller BR.** 2012. Infection and transmission of Rift Valley fever viruses lacking the NSs and/or NSm genes in mosquitoes: potential role for NSm in mosquito infection. PLoS Negl. Trop. Dis. **6**e1639. doi:10.1371/journal.pntd.0001639.
11. **Won S, Ikegami T, Peters CJ, Makino S.** 2007. NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. J. Virol. **81**:13335–13345.
12. **Bird BH, Albarino CG, Nichol ST.** 2007. Rift Valley fever virus lacking NSm proteins retains high virulence in vivo and may provide a model of human delayed onset neurologic disease. Virology **362**:10–15.
13. **Ikegami T, Won S, Peters CJ, Makino S.** 2006. Rescue of infectious Rift Valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. J. Virol. **80**:2933–2940.
14. **Kaput J, Brandriss MC, Prussak-Wieckowska T.** 1989. In vitro import of cytochrome c peroxidase into the intermembrane space: release of the processed form by intact mitochondria. J. Cell Biol. **109**:101–112.
15. **Neubig RR, Krodel EK, Boyd ND, Cohen JB.** 1979. Acetylcholine and local anesthetic binding to Torpedo nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc. Natl. Acad. Sci. U. S. A. **76**:690–694.
16. **Hofmann K, Stoffel W.** 1993. TMbase—a database of membrane spanning protein segments. Biol. Chem. Hoppe-Seyler **374**:166.
17. **Horie C, Suzuki H, Sakaguchi M, Mihara K.** 2002. Characterization of signal that directs C-tail-anchored proteins to mammalian mitochondrial outer membrane. Mol. Biol. Cell **13**:1615–1625.
18. **Kanaji S, Iwahashi J, Kida Y, Sakaguchi M, Mihara K.** 2000. Characterization of the signal that directs Tom20 to the mitochondrial outer membrane. J. Cell Biol. **151**:277–288.
19. **Kaufmann T, Schlipf S, Sanz J, Neubert K, Stein R, Borner C.** 2003. Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. J. Cell Biol. **160**:53–64.
20. **Desbien AL, Kappler JW, Marrack P.** 2009. The Epstein-Barr virus Bcl-2 homolog, BHRF1, blocks apoptosis by binding to a limited amount of Bim. Proc. Natl. Acad. Sci. U. S. A. **106**:5663–5668.
21. **Han J, Sabbatini P, Perez D, Rao L, Modha D, White E.** 1996. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev. **10**:461–477.
22. **Loh J, Huang Q, Petros AM, Nettesheim D, van Dyk LF, Labrada L, Speck SH, Levine B, Olejniczak ET, Virgin HW.** 2005. A surface groove essential for viral Bcl-2 function during chronic infection in vivo. PLoS Pathog. **1**e10. doi:10.1371/journal.ppat.0010010.
23. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods **9**:671–675.
24. **Bozidis P, Williamson CD, Colberg-Poley AM.** 2007. Isolation of

endoplasmic reticulum, mitochondria, and mitochondria-associated membrane fractions from transfected cells and from human cytomegalovirus-infected primary fibroblasts. Curr. Protoc. Cell Biol. **3**73.27.1–3.27.23.
25. **Green N, Fang H, Kalies KU, Canfield V.** 2001. Determining the topology of an integral membrane protein. Curr. Protoc. Cell Biol. **3**15.2.1–5.2.27.
26. **Schwer B, Ren S, Pietschmann T, Kartenbeck J, Kaehlcke K, Bartenschlager R, Yen TS, Ott M.** 2004. Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. J. Virol. **78**:7958–7968.
27. **Eskes R, Desagher S, Antonsson B, Martinou JC.** 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol. Cell. Biol. **20**:929–935.
28. **Kyte J, Doolittle RF.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.